

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|   |           |  |
|---|-----------|--|
| (51) International Patent Classification 6:<br><b>C12S 3/14</b>   | <b>A1</b> | (11) International Publication Number: <b>WO 96/27027</b><br>(43) International Publication Date: 6 September 1996 (06.09.96)  |
| <p>(21) International Application Number: PCT/US96/02797</p> <p>(22) International Filing Date: 1 March 1996 (01.03.96)</p> <p>(30) Priority Data: 08/398,552 2 March 1995 (02.03.95) US</p> <p>(71) Applicant: RUTGERS THE STATE UNIVERSITY OF NEW JERSEY (US/US); Administrative Service Building, Annex II, Busch Campus, Piscataway, NJ 08855 (US).</p> <p>(72) Inventor: WARD, William; 149 E. Chestnut Street, Metuchen, NJ 08840 (US).</p> <p>(74) Agent: BEHR, Omri, M.; 325 Pierson Avenue, Edison, NJ 08837 (US).</p>   |           | <p>(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p><b>Published</b><br/><i>With international search report.<br/>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> |
| <p>(54) Title: IMPROVED METHOD FOR PURIFYING GREEN FLUORESCENT PROTEIN</p>  |           |  |
| <p>(57) Abstract</p> <p>An improved method for purifying GFP from other components in a biological sample comprising, subjecting said sample to means for isolating GFP under conditions favoring GFP in monomer form; and in another step, subjecting said sample to means for isolating GFP under conditions favoring GFP in dimer form. Suitably said means for separating is a hydrophobic interaction chromatography column and the conditions favoring GFP in monomer and dimer form are low GFP (below 5 mg/ml) and salt concentrations and high GFP (over 5 mg/ml) and salt concentrations, respectively.</p> |           |  |

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                          |    |                                       |    |                          |
|----|--------------------------|----|---------------------------------------|----|--------------------------|
| AM | Armenia                  | GB | United Kingdom                        | MW | Malawi                   |
| AT | Austria                  | GE | Georgia                               | MX | Mexico                   |
| AU | Australia                | GN | Guinea                                | NE | Niger                    |
| BB | Barbados                 | GR | Greece                                | NL | Netherlands              |
| BE | Belgium                  | HU | Hungary                               | NO | Norway                   |
| BF | Burkina Faso             | IE | Ireland                               | NZ | New Zealand              |
| BG | Bulgaria                 | IT | Italy                                 | PL | Poland                   |
| BJ | Benin                    | JP | Japan                                 | PT | Portugal                 |
| BR | Brazil                   | KE | Kenya                                 | RO | Romania                  |
| BY | Belarus                  | KG | Kyrgyzstan                            | RU | Russian Federation       |
| CA | Canada                   | KP | Democratic People's Republic of Korea | SD | Sudan                    |
| CF | Central African Republic | KR | Republic of Korea                     | SE | Sweden                   |
| CG | Congo                    | KZ | Kazakhstan                            | SG | Singapore                |
| CH | Switzerland              | LJ | Liechtenstein                         | SI | Slovenia                 |
| CI | Côte d'Ivoire            | LK | Sri Lanka                             | SK | Slovakia                 |
| CM | Cameroon                 | LR | Liberia                               | SN | Senegal                  |
| CN | China                    | LT | Lithuania                             | SZ | Swaziland                |
| CS | Czechoslovakia           | LU | Luxembourg                            | TD | Chad                     |
| CZ | Czech Republic           | LV | Latvia                                | TG | Togo                     |
| DE | Germany                  | MC | Monaco                                | TJ | Tajikistan               |
| DK | Denmark                  | MD | Republic of Moldova                   | TT | Trinidad and Tobago      |
| EE | Estonia                  | MG | Madagascar                            | UA | Ukraine                  |
| ES | Spain                    | ML | Mali                                  | UG | Uganda                   |
| FI | Finland                  | MN | Mongolia                              | US | United States of America |
| FR | France                   | MR | Mauritania                            | UZ | Uzbekistan               |
| GA | Gabon                    |    |                                       | VN | Viet Nam                 |

## IMPROVED METHOD FOR PURIFYING GREEN FLUORESCENT PROTEIN

### FIELD OF THE INVENTION

5        This invention concerns an improved method for purifying green fluorescent protein, whereby the speed of purifying green fluorescent protein to high purity and yield is dramatically improved.

### BACKGROUND OF THE INVENTION

10        Green fluorescent protein ("GFP") is a 27,000 dalton fluorescent polypeptide found in numerous marine organisms, notably the jellyfish *Aequorea victoria*. The fluorescence of GFP is due to the presence of a chromophore formed from residues 65 through 67 in the protein.

15        GFP reversibly dimerizes into a 54,000 dalton dimer. The interaction between GFP molecules resulting in dimerization is believed to be a hydrophobic interaction; and the dimer does exhibit lower hydrophobicity than the monomer. GFP dimers are found at relatively high concentrations of GFP in aqueous solution at pH 7 (higher than 5 mg/ml), whereas the  
20        monomer form prevails at lower concentrations (at or below about 5 mg/ml).

      GFP is present at such extremely low levels *in vivo* that investigators in the field of bioluminescence have typically gathered tens of thousands of the organisms which synthesize GFP in order to isolate GFP in gram or  
25        milligram amounts. Purification of these extremely low levels of GFP has been a burdensome and lengthy task. Biological samples derived from GFP-synthesizing organisms are subjected to conventional purification techniques, such as centrifugation, chromatography and gel electrophoresis. However, due to the low levels of GFP in the samples, these steps must be  
30        performed with great care to avoid GFP loss. This requires repeated passage through large chromatography columns, a task made quite slow due to the viscosity of biological samples. As a result, purification of GFP to

high levels is a task which has conventionally required at least several weeks and frequently several months.

In recent years, the gene for *A. victoria* GFP has been isolated and  
5 success-fully expressed in a variety of procaryotic and eucaryotic organisms.  
Several GFP synthetic analogs have been developed from this gene,  
including the modified GFP pre-coelenterazine peptides described in PCT US  
95/01425 by Ward et al., filed February 3, 1995 (in which the native Ser  
residue 65 is replaced with Tyr, and other residues are replaceable or  
10 deleted), incorporated herein by reference. Other GFP analogs are the GFP  
mutant peptides exhibiting non-green fluorescence described in Hime et al.,  
Proc.Nat.Acad.Sci 91 12501-12504 (1994), and Delagrave et al.,  
Biotechnology 13, 151-154, 13 February 1995, both incorporated herein by  
reference.

15

Employing this GFP gene, or a mutant variant thereof, permits one to  
synthesize GFP or GFP analogs in higher amounts than are found in nature,  
and so reduces to some extent the difficulties of purifying these compounds;  
with larger biological samples, the care needed to avoid GFP loss is no  
20 longer so acute. Nevertheless, the problem of purifying GFP from other  
compounds in a biological sample still remains, particularly where one  
wishes to reach high purity levels on the order of 95 to 98% purity.

#### SUMMARY OF THE INVENTION

25 Applicants have discovered that by arraying conventional purification  
techniques in a novel manner so as to exploit the ability of GFP to dimerize  
reversibly, they are able to isolate GFP in higher levels of purity and in  
dramatically less time than by methods employed to date.

30 Accordingly, there is provided a method of purifying GFP from a  
biological sample, said method comprising, in one step, subjecting the  
sample to means for separating GFP from the other sample components

under conditions favoring GFP in the monomer form; and, in another step, subjecting the sample to means for separating GFP from the other sample components under conditions favoring GFP in the dimer form. Either step may be performed first, with the fraction of the sample containing GFP which results from that step then being subjected to the other step. This method is useful for purifying GFP produced by naturally bioluminescing organisms, or by organisms transformed with the GFP gene, as well as GFP analogs.

10 In this method, the biological sample may be subjected to a first purification step in which GFP is present at a low concentration of 0.01 to 1, or 0.2 to 0.5 mg/ml and a second purification step in which GFP is present at a high concentration of 5 to 200 or 20 to 200 mg/ml.

15 The means for separating GFP from the other sample components include chromatographic, centrifugation, ultrafiltration and electrophoretic techniques, as well as other techniques known to persons having skill in the art. More particularly, the chromatographic techniques which may be employed include high pressure liquid chromatography, affinity  
20 chromatography, size exclusion chromatography and hydrophobic interaction chromatography. Thus, in one embodiment, the means for separating GFP from the other sample components are chromatographic columns selected from the group consisting of hydrophobic interaction and size exclusion chromatography columns.

25

Suitably, where the means for separating is a hydrophobic interaction chromatography column, the step of subjecting GFP to separation from the other components under conditions favoring the monomer form is performed prior to the step favoring GFP in the dimer form. Thus, the first step may  
30 be run with GFP at low concentration and the second step with GFP at high concentration.

In one embodiment, there is thus provided a method for purifying GFP from a biological sample which comprises subjecting this sample to two separate passages through a hydrophobic interaction chromatography column. One of the passages is performed under conditions favoring GFP in monomer form and the other under conditions favoring GFP in dimer form. These conditions favoring GFP in monomer or dimer form may comprise GFP being present in said biological sample at a concentration of 0.2 to 0.5 mg/ml, or at 20 to 200 mg/ml, respectively.

10 An unusually high degree of purification is achieved by this method because the eluting conditions for GFP dimer in the second passage are strong binding conditions for monomer in the first passage. Thus, only contaminants that behave in a similar way (i.e., which lose their hydrophobicity upon dimerization) will be expected to co-elute with GFP.

15 The chromatographic step in which the GFP is in monomer form may be performed either before or following the step in which GFP is a dimer. Suitably, the passage in which GFP is in monomer form is performed prior to the passage under conditions favoring GFP in dimer form.

20 This novel method for purifying GFP applies equally well to analogs of GFP known to persons skilled in the art. Thus there is further provided a method for purifying a GFP analog from a biological sample comprising subjecting the sample to two separate passages through a hydrophobic interaction chromatography column. One of the passages is performed under conditions favoring the GFP analog in monomer form and the other passage favoring the GFP analog in dimer form.

Employing this embodiment, a biological sample comprising GFP derived from *A. victoria* according to methods well known to those skilled in the art is subjected to two passages through hydrophobic interaction chromatography columns. In the first passage, the sample is dilute (GFP

concentration 0.1 to 5 mg/ml) to favor GFP being in monomer form. Consequently, the GFP adheres to the hydrophobic interaction chromatography column matrix while non-hydrophobic components of the sample pass through the column. Following an isocratic wash (1-5 column  
5 volumes), the GFP is removed from the matrix by lowering the salt concentration abruptly in order to reduce the hydrophobic interaction between the matrix and the GFP monomer. This causes all the GFP to be released from the matrix and to elute in a narrow band. The GFP-containing fraction which elutes from this first passage no longer contains the original  
10 non-hydrophobic components. This first passage through a chromatograph column may be completed in several hours.

The GFP-containing fraction resulting from the first passage is next concentrated in order to dimerize the GFP. The dimer form is maintained  
15 by keeping the GFP concentration relatively high (*i.e.*, 10 to 200 mg/ml). In dimer form, the GFP has less hydrophobicity, and hence less hydrophobic interaction with a hydrophobic matrix. This fraction is therefore passed through a second hydrophobic interaction chromatography column under higher salt concentration in order to cause the GFP dimer to bind to the  
20 matrix. Suitably, this second column contains the same hydrophobic interaction matrix material as the first column in a smaller volume than the first in order to maintain high concentration of GFP, and hence GFP in dimer form. The hydrophobic components of the fraction, including the GFP dimers, bind to the matrix, while less hydrophobic components again pass  
25 through the column. To separate the GFP dimer from the matrix, the carrier salt concentration is lowered, suitably from 1.5 M to 1.0 M  $(\text{NH}_4)_2\text{SO}_4$ , thus releasing the dimerized GFP from the matrix to elute in a narrow band.

This lowering of the salt concentration however releases little else  
30 from the matrix besides the GFP dimer. The dimer is released at this salt concentration because it exhibits different hydrophobicity than the monomer. However, nearly all the remaining sample components which

bound to the matrix at 0.5 M in the first column and eluted with GFP will not be released from the second column. Accordingly, the GFP released in dimer form from the second column is highly pure.

5        This purification method successfully purifies GFP or GFP analogs to 90% levels in hours or days rather than weeks or months. One may wish to perform preliminary steps to remove undesirable components from the biological sample or further steps subsequent to purification to further increase purity. Even with these optional steps, the purification method  
10 generally requires no more than three to five days.

#### DETAILED DESCRIPTION OF THE INVENTION

The biological samples from organisms synthesizing GFP, or an analog thereof, are derived by methods well known to those skilled in the art.  
15 Suitable materials for use as hydrophobic interaction chromatography matrices include octyl, hexyl, and phenyl agarose with 3-carbon long spacer arms. Suitable materials for use as size exclusion chromatography matrices include Biogel P-60 and P-100; and Sephadex G-75 and G-100. The purity of the final GFP, or GFP analog, may be measured by methods well known  
20 to those skilled in the art, including absorption spectroscopy and electrophoretic analyses.

Protease inhibitors used early in purification help to reduce the extent of C-terminal truncation (1-8 amino acid residues), which might otherwise  
25 occur during purification of both native and recombinant GFP. Such limited truncation produces isoelectric variants of GFP that differ insignificantly in most physical properties. These isoelectric variants are considered to be GFP analogs for purposes of this invention.

30        If it is necessary to eliminate minor isoforms of pure GFP, FPLC on Mono Q and native gel electrophoresis on a Bio Rad Prep Cell have been shown to be effective.



In one preferred embodiment, the method of purifying GFP comprises subjecting a biological sample to size exclusion chromatography in one molar buffered ammonium sulfate; subjecting the eluate of such size exclusion chromatography to hydrophobic interaction chromatography using pre-column batch adsorption in one molar ammonium sulfate and eluting the column with a decreasing salt gradient (1.0 to 0 molar ammonium sulfate); subjecting the eluate of this hydrophobic interaction chromatography to ammonium sulfate precipitation in order to concentrate the eluate, followed by redissolving the precipitate in 1.5 M ammonium sulfate; subjecting the redissolved eluate to a second hydrophobic interaction chromatography run at high protein concentration in 1.5 M  $(\text{NH}_4)_2\text{SO}_4$  and eluting in a 1.5 to 1 M decreasing salt gradient; and concentrating the eluate by ultrafiltration to the highest protein concentration attainable (generally greater than 50 mg/ml). Suitably the ultrafiltration is performed in ammonium sulfate at moderate to high concentration (e.g., 0.5 M) in order to maintain the dimer form. The dimer, thus isolated, may be further purified by subjecting it to size exclusion chromatography on P-100 Biogel column (1 x 120 cm). The upper 20 percent of the column may be equilibrated with 0.5 M ammonium sulfate, again to help maintain the GFP in dimer form.

20

### EXAMPLE

#### *Details of Purification for Recombinant GFP*

1. Cell Disruption. In order to prepare a biological sample, a culture of *E. coli* cells transformed with the *A. victoria* GFP gene prepared as described in Chalfie et al. Science **263**, 802-805 (1994) (incorporated herein by reference) is sonicated following 1 hour treatment with lysozyme at pH 7.0 at 0°C. PMSF, pepstatin and other protease inhibitors are incorporated in the lysozyme buffer to prevent truncation of C-terminal amino acid residues 230-238.

30

## 2. Batch purification.

a. The sonicate from the cell disruption step is subjected to centrifugation at 10,000 x g for 20 minutes to remove cellular debris.

5      b. The supernatant of this centrifugation is subjected to precipitation of nucleic acids with protamine sulfate, followed by centrifugation at 10,000 x g for 20 minutes. Protamine sulfate concentration is determined with every batch by small scale titration with 100  $\mu$ l portions of cell sonicate.

10

c. Ammonium sulfate precipitation. The supernatant from the protamine sulfate treatment is precipitated with  $(\text{NH}_4)_2\text{SO}_4$  to 75% of saturation at pH 7.5 and 0°C. After centrifugation at 10,000 x g for 20 minutes, the pellet is collected and the supernatant is discarded. The  
15  $(\text{NH}_4)_2\text{SO}_4$  pellet is redissolved in 300-600 ml of 10 mM Tris-EDTA buffer pH 7.5, 0.02%  $\text{NaN}_3$ , with  $(\text{NH}_4)_2\text{SO}_4$  at 25% of saturation (about 1.0 M) containing a protease inhibitor cocktail of PMSF, pepstatin, and suitably other inhibitors. The suspension is centrifuged at 10,000 x g for 30 minutes to remove insoluble material.

20

## 3. P-100 Biogel Gel Filtration (Monomer Conditions)

The  $(\text{NH}_4)_2\text{SO}_4$  supernatant (25% saturation) from batch purification is chromatographed on an 8 liter column (column dimensions, 10 cm x 120 cm) of P-100 Biogel (medium) in 10 mM Tris-EDTA and 0.02%  $\text{NaN}_3$ .  
25 Usually, 200 ml - 600 ml samples are applied. The low GFP concentration here favors GFP being in monomer form. In this step, GFP chromatographs primarily as a monomer (MW 27,000). If the GFP concentration is kept below 0.2 mg/ml in the sample applied to the column (40 mg to 120 mg total GFP), the monomer form is assured. Somewhat higher concentrations,  
30 however, up to 0.5 mg/ml, appear to be acceptable (100 mg -300 mg total GFP).

GFP is found to elute as a monomer with apparent molecular weight of 21,000 daltons lower because of slight hydrophobic interaction between the monomeric GFP and the column matrix. This hydrophobic retardation is promoted by high salt (1 M  $(\text{NH}_4)_2\text{SO}_4$ ) and produces a population of 5 molecules, co-chromatographing with GFP, that have a lower average molecular weight than 27,000 daltons. The existence of this co-chromatographing population renders the second hydrophobic interaction chromatography step particularly important. The eluted portions are pooled asymmetrically to favor the lower molecular weight portion of the peak, *i.e.*, 10 the later eluting fractions.

#### 4. Hydrophobic Interaction Chromatography on Octyl Agarose (Monomer conditions)

The pooled GFP sample from P-100 is precipitated with  $(\text{NH}_4)_2\text{SO}_4$ , 15 (100% saturation) and resuspended in 1.0 M  $(\text{NH}_4)_2\text{SO}_4$  (total volume approximately 200 ml) in 10 mM Tris-EDTA buffer with 0.02%  $\text{NaN}_3$ , pH 7.5. To this 200 ml sample is added 50 ml of settled octyl agarose also equilibrated in 1.0 M  $(\text{NH}_4)_2\text{SO}_4$  in 10 mM Tris-EDTA buffer at pH 7.5 containing 0.02%  $\text{NaN}_3$ . The 250 ml slurry is applied to a column of octyl 20 agarose (5cm x 20 cm) containing a 150 ml packet bed of octyl agarose equilibrated in the same buffer. The pre-column batch adsorption step is incorporated so as to distribute the bound GFP over a wide surface area of octyl agarose. This keeps the GFP concentration low enough to favor monomer binding of GFP to the stationary phase.

25

The column is washed with 1 column volume of 1.0 M  $(\text{NH}_4)_2\text{SO}_4$  buffer (200 ml) followed by a sufficient volume of 0.5 M  $(\text{NH}_4)_2\text{SO}_4$  buffer (several column volumes) to promote elution. After about 5% of the total GFP has eluted, the eluting buffer is switched to 10 mM Tris-EDTA, 0.02% 30  $\text{NaN}_3$ , pH 7.5 with no  $(\text{NH}_4)_2\text{SO}_4$ . This buffer switch promotes rapid elution from the column of all of the bound GFP in a small concentrated fraction ( $\leq$  50ml).

### 5. Hydrophobic Interaction Chromatography on Octyl Agarose (Dimer Conditions)

The pooled GFP from the preceding step is adjusted to 1.5 M  $(\text{NH}_4)_2\text{SO}_4$  with a saturated solution (4M  $(\text{NH}_4)_2\text{SO}_4$ ) of stock buffer. The  
5 stock 4 M  $(\text{NH}_4)_2\text{SO}_4$  is slowly added up to, but not beyond, the range required to precipitate GFP. This sample is loaded directly onto a small octyl agarose column (2 cm x 20 cm) containing a 50% excess of octyl agarose needed to bind all the GFP. Thus, the GFP sample should penetrate approximately 2/3 of the column distance when all has been loaded. The  
10 column is then washed with 1.0 M  $(\text{NH}_4)_2\text{SO}_4$  (in the usual stock buffer) until all the GFP has eluted. Suitably, this column has a small bed volume so that there is no significant GFP dilution to drive GFP dimers into the monomer form.

15 An unusually high degree of purification is achieved in this step, because the eluting conditions for GFP dimer (1.0 M  $(\text{NH}_4)_2\text{SO}_4$ ) are strong binding conditions for monomer. Thus, only contaminants that behave in a similar way (i.e., which lose their hydrophobicity upon dimerization) will be expected to co-elute with GFP.

20

### 6. P-100 Biogel gel filtration (Dimer Conditions)

The sample of GFP eluting from the second hydrophobic interaction chromatography column may be further concentrated by ultrafiltration and washed on the membrane with 10 mM Tris-EDTA pH 7.5 or 8.0  $\pm$  0.02%  
25  $\text{NaN}_3$ . Alternatively, 10 mM sodium phosphate pH 7.0 buffer may be used. EDTA is not essential. Moderate concentrations of ammonium sulfate (0.5 M) may be used to favor dimer formation.

The sample is concentrated to the point of precipitation (e.g., to  
30 approximately 200 mg/ml) and clarified by microcentrifugation. The concentrated sample is applied to a 1 x 120 cm column of P-100 BioGel (fine). Sample volume is approximately 3 ml (slightly greater than 2 to 3%)

of column volume). Smaller volumes result in greater dilution and movement toward monomer.

The purity of the final product is judged by electrophoretic analyses 5 and absorption spectroscopy. The quotient  $A_{395} / A_{280}$  value of  $\geq 1.2$  indicates  $\geq 98\%$  purity.

CLAIMS

1. A method for purifying GFP from a biological sample, said method comprising subjecting said sample to two separate passages  
5 through a hydrophobic interaction chromatography column, wherein one of said passages is performed under conditions favoring GFP in monomer form and the other passage is performed under conditions favoring GFP in dimer form.

10 2. The method according to Claim 1, wherein the conditions favoring GFP in monomer form comprise GFP being present in said biological sample at a concentration of 0.2 to 0.5 mg/ml.

3. The method according to Claim 2, wherein the conditions  
15 favoring GFP in dimer form comprise GFP being present in said biological sample at a concentration of of 20 to 200mg/ml.

4. The method according to Claim 3, wherein said passage  
performed under conditions favoring GFP in monomer form is performed  
20 prior to said passage under conditions favoring GFP in dimer form.

5. A method for purifying a GFP analog from a biological sample, said method comprising subjecting said sample to two separate passages  
through a hydrophobic interaction chromatography column, wherein one of  
25 said passages is performed under conditions favoring GFP in monomer form and the other passage is performed under conditions favoring GFP in dimer form.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/02797

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12S 3/14

US CL : 530/413

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/413

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| X         | Dissertation submitted to the Graduate School - New Brunswick Rutgers, The State University of New Jersey, published October 1985, Amy F. Roth, "Purification and Protease Susceptibility of the Green-Fluorescent Protein of Aequorea Aequorea with a Note on Halostaura", whole document, especially pages 1-17 and 24-38. | 1-5                   |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| Special categories of cited documents:   |  |
|--|--|
| *A* document defining the general state of the art which is not considered to be part of particular relevance  | *T* later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention   |
| *E* earlier document published on or after the international filing date   | *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone   |
| *L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *O* document referring to an oral disclosure, use, exhibition or other means   | *Z* document of member of the same patent family   |
| *P* document published prior to the international filing date but later than the priority date claimed   |  |

Date of the actual completion of the international search

18 JUNE 1996

Date of mailing of the international search report

02 JUL 1996

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

PATRICK DELANEY

Telephone No. (703) 308-0196